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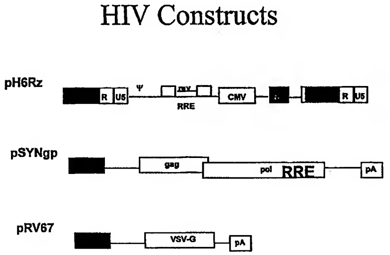

(57) Abstract

A viral vector production system is provided which system comprises: (i) a viral genome comprising at least one first nucleotide sequence encoding a gene product capable of binding to and effecting the cleavage, directly or indirectly, of a second nucleotide sequence, or transcription product thereof, encoding a viral polypeptide required for the assembly of viral particles; (ii) a third nucleotide sequence encoding said viral polypeptide required for the assembly of the viral genome into viral particles, which third nucleotide sequence has a different nucleotide sequence to the second nucleotide sequence such that said third nucleotide sequence, or transcription product thereof, is resistant to cleavage directed by said gene product. The viral vector production system may be used to produce viral particles for use in treating or preventing viral infection.

ANTI-VIRAL VECTORS

Field of the Invention

The present invention relates to novel viral vectors capable of delivering anti-viral inhibitory RNA molecules to target cells.

Background to the Invention

The application of gene therapy to the treatment of AIDS and HIV infection has been discussed widely (14). The types of therapeutic gene proposed usually fall into one of two broad categories. In the first the gene encodes protein products that inhibit the virus in a number of possible ways. One example of such a protein is the RevM10 derivative of the HIV Rev protein (16). The RevM10 protein acts as a transdominant negative mutant and so competitively inhibits Rev function in the virus. Like many of the protein-based strategies, the RevM10 protein is a derivative of a native HIV protein. While this provides the basis for the anti-HIV effect, it also has serious disadvantages. In particular, this type of strategy demands that in the absence of the virus there is little or no expression of the gene. Otherwise, healthy cells harbouring the gene become a target for the host cytotoxic T lymphocyte (CTL) system, which recognises the foreign protein (17, 25). The second broad category of therapeutic gene circumvents these CTL problems. The therapeutic gene encodes inhibitory RNA molecules; RNA is not a target for CTL recognition. The RNA molecules may be anti-sense RNA (15, 31), ribozymes (5) or competitive decoys (1).

Ribozymes are enzymatic RNA molecules which catalyse sequence-specific RNA processing. The design and structure of ribozymes has been described extensively in the literature in recent years (3, 7, 31). Amongst the most powerful systems are those that deliver multitarget ribozymes that cleave RNA of the target virus at multiple sites (5, 21).

In recent years a number of laboratories have developed retroviral vector systems based on HIV (2, 4, 18, 19, 22-24, 27, 32, 35, 39, 43). In the context of anti-HIV gene therapy these vectors have a number of advantages over the more conventional murine based vectors

such as murine leukaemia virus (MLV) vectors. Firstly, HIV vectors would target precisely those cells that are susceptible to HIV infection (22, 23). Secondly, the HIV-based vector would transduce cells such as macrophages that are normally refractory to transduction by murine vectors (19, 20). Thirdly, the anti-HIV vector genome would be propagated through the CD4+ cell population by any virus (HIV) that escaped the therapeutic strategy (7). This is because the vector genome has the packaging signal that will be recognised by the viral particle packaging system. These various attributes make HIV-vectors a powerful tool in the field of anti-HIV gene therapy.

A combination of the multitarget ribozyme and an HIV-based vector would be attractive as a therapeutic strategy. However, until now this has not been possible. Vector particle production takes place in producer cells which express the packaging components of the particles and package the vector genome. The ribozymes that are designed to destroy the viral RNA would therefore also interrupt the expression of the components of the HIV-based vector system during vector production. The present invention aims to overcome this problem.

Summary of the Invention

It is therefore an object of the invention to provide a system and method for producing viral particles, in particular HIV particles, which carry nucleotide constructs encoding inhibitory RNA molecules such as ribozymes and/or antisense RNAs directed against a corresponding virus, such as HIV, within a target cell, that overcomes the above-mentioned problems. The system includes both a viral genome encoding the inhibitory RNA molcules and nucleotide constructs encoding the components required for packaging the viral genome in a producer cell. However, in contrast to the prior art, although the packaging components have substantially the same amino acid sequence as the corresponding components of the target virus, the inhibitory RNA molecules do not affect production of the viral particles in the producer cells because the nucleotide sequence of the packaging components used in the viral system have been modified to prevent the inhibitory RNA molecules from effecting cleavage or degradation of the RNA transcripts produced from the constructs. Such a viral particle may be used to treat viral infections, in

particular HIV infections.

Accordingly the present invention provides a viral vector system comprising:

(i) a first nucleotide sequence encoding a gene product capable of binding to and effecting the cleavage, directly or indirectly, of a second nucleotide sequence, or transcription product thereof, encoding a viral polypeptide required for the assembly of viral particles; and

(ii) a third nucleotide sequence encoding said viral polypeptide required for the assembly of viral particles, which third nucleotide sequence has a different nucleotide sequence to the second nucleotide sequence such that the third nucleotide sequence, or transcription product thereof, is resistant to cleavage directed by said gene product.

In another aspect, the present invention provides a viral vector production system comprising:

(i) a viral genome comprising at least one first nucleotide sequence encoding a gene product capable of binding to and effecting the cleavage, directly or indirectly, of a second nucleotide sequence, or transcription product thereof, encoding a viral polypeptide required for the assembly of viral particles;

(ii) a third nucleotide sequence encoding said viral polypeptide required for the assembly of the viral genome into viral particles, which third nucleotide sequence has a different nucleotide sequence to the second nucleotide sequence such that said third nucleotide sequence, or transcription product thereof, is resistant to cleavage directed by said gene product.

The gene product is typically an RNA inhibitory sequence selected from a ribozyme and an anti-sense ribonucleic acid, preferably a ribozyme.

Preferably, the viral vector is a retroviral vector, more preferably a lentiviral vector, such as an HIV vector. The second nucleotide sequence and the third nucleotide sequences are typically from the same viral species, more preferably from the same viral strain. Generally, the viral genome is also from the same viral species, more preferably from the same viral strain.

In the case of retroviral vectors, the polypeptide required for the assembly of viral particles is selected from gag, pol and env proteins. Preferably at least the gag and pol sequences are lentiviral sequences, more preferably HIV sequences. Alternatively, or in addition, the env sequence is a lentiviral sequence, more preferably an HIV sequence.

In a preferred embodiment, the third nucleotide sequence is resistant to cleavage directed by the gene product as a result of one or more conservative alterations in the nucleotide sequence which remove cleavage sites recognised by the at least one gene product and/or binding sites for the at least one gene product. For example, where the gene product is a ribozyme, the third nucleotide sequence is adapted to be resistant to cleavage by the ribozyme.

Preferably the third nucleotide sequence is codon optimised for expression in host cells. The host cells, which term includes producer cells and packaging cells, are typically mammalian cells.

In a particularly preferred embodiment, (i) the viral genome is an HIV genome comprising nucleotide sequences encoding anti-HIV ribozymes and/or anti-HIV antisense sequences directed against HIV packaging component sequences (such as gag.pol) in a target HIV and (ii) the viral system for producing packaged HIV particles further comprises nucleotide constructs encoding the same packaging components (such as gag.pol proteins) as in the target HIV wherein the sequence of the nucleotide constructs is different from that found in the target HIV so that the anti-HIV ribozyme and/or antisense HIV sequences cannot effect cleavage or degradation of the gag.pol transcripts during production of the HIV particles in producer cells.

The present invention also provides a viral particle comprising a viral vector according to the present invention and one or more polypeptides encoded by the third nucleotide sequences according to the present invention. For example the present invention provides a viral particle produced using the viral vector production system of the invention.

In another aspect, the present invention provides a method for producing a viral particle which method comprises introducing into a host cell (i) a viral genome vector according to the present invention; (ii) one or more third nucleotide sequences according to the present invention; and (iii) nucleotide sequences encoding the other essential viral packaging components not encoded by the one or more third nucleotide sequences.

The present invention further provides a viral particle produced using by the method of the invention.

The present invention also provides a pharmaceutical composition comprising a viral particle according to the present invention together with a pharmaceutically acceptable carrier or diluent.

The viral system of the invention or viral particles of the invention may be used to treat viral infections, particularly retroviral infections such as lentiviral infections including HIV infections. Thus the present invention provides a method of treating a viral infection which method comprises administering to a human or animal patient suffering from the viral infection an effective amount of a viral system, viral particle or pharmaceutical composition of the present invention.

The invention relates in particular to HIV-based vectors carrying anti-HIV ribozymes. However, the invention can be applied to any other virus, in particular any other lentivirus, for which treatment by gene therapy may be desirable. The invention is illustrated herein for HIV, but this is not considered to limit the scope of the invention to HIV-based anti-HIV vectors.

Detailed Description of the Invention

The term "viral vector" refers to a nucleotide construct comprising a viral genome capable of being transcribed in a host cell, which genome comprises sufficient viral genetic information to allow packaging of the viral RNA genome, in the presence of packaging components, into a viral particle capable of infecting a target cell. Infection of the target

cell includes reverse transcription and integration into the target cell genome, where appropriate for particular viruses. The viral vector in use typically carries heterologous coding sequences (nucleotides of interest) which are to be delivered by the vector to the target cell, for example a first nucleotide sequence encoding a ribozyme. A viral vector is incapable of independent replication to produce infectious viral particles within the final target cell.

The term " viral vector system" is intended to mean a kit of parts which can be used when combined with other necessary components for viral particle production to produce viral particles in host cells. For example, the first nucleotide sequence may typically be present in a plasmid vector construct suitable for cloning the first nucleotide sequence into a viral genome vector construct. When combined in a kit with a third nucleotide sequence, which will also typically be present in a separate plasmid vector construct, the resulting combination of plasmid containing the first nucleotide sequence and plasmid containing the third nucleotide sequence comprises the essential elements of the invention. Such a kit may then be used by the skilled person in the production of suitable viral vector genome constructs which when transfected into a host cell together with the plasmid containing the third nucleotide sequence, and optionally nucleic acid constructs encoding other components required for viral assembly, will lead to the production of infectious viral particles.

Alternatively, the third nucleotide sequence may be stably present within a packaging cell line that is included in the kit.

The kit may include the other components needed to produce viral particles, such as host cells and other plasmids encoding essential viral polypeptides required for viral assembly. By way of example, the kit may contain (i) a plasmid containing a first nucleotide sequence encoding an anti-HIV ribozyme and (ii) a plasmid containing a third nucleotide sequence encoding a modified HIV gag.pol construct which cannot be cleaved by the anti-HIV ribozyme. Optional components would then be (a) an HIV viral genome construct with suitable restriction enzyme recognition sites for cloning the first nucleotide sequence into the viral genome; (b) a plasmid encoding a VSV-G env protein. Alternatively, nucleotide

sequence encoding viral polypeptides required for assembly of viral particles may be provided in the kit as packaging cell lines comprising the nucleotide sequences, for example a VSV-G expressing cell line.

The term "viral vector production system" refers to the viral vector system described above wherein the first nucleotide sequence has already been inserted into a suitable viral vector genome.

Viral vectors are typically retroviral vectors, in particular lentiviral vectors such as HIV vectors. The retroviral vector of the present invention may be derived from or may be derivable from any suitable retrovirus. A large number of different retroviruses have been identified. Examples include: murine leukemia virus (MLV), human immunodeficiency virus (HIV), simian immunodeficiency virus, human T-cell leukemia virus (HTLV). equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV). A detailed list of retroviruses may be found in Coffin *et al.,* 1997, "Retroviruses", Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763.

Details on the genomic structure of some retroviruses may be found in the art. By way of example, details on HIV and Mo-MLV may be found from the NCBI Genbank (Genome Accession Nos. AF033819 and AF033811, respectively).

The lentivirus group can be split even further into "primate" and "non-primate". Examples of primate lentiviruses include human immunodeficiency virus (HIV), the causative agent of human auto-immunodeficiency syndrome (AIDS), and simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

The basic structure of a retrovirus genome is a 5' LTR and a 3' LTR, between or within which are located a packaging signal to enable the genome to be packaged, a primer binding site, integration sites to enable integration into a host cell genome and *gag, pol* and *env* genes encoding the packaging components - these are polypeptides required for the assembly of viral particles. More complex retroviruses have additional features, such as *rev* and RRE sequences in HIV, which enable the efficient export of RNA transcripts of the integrated provirus from the nucleus to the cytoplasm of an infected target cell.

In the provirus, these genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. LTRs also serve as enhancer-promoter sequences and can control the expression of the viral genes. Encapsidation of the retroviral RNAs occurs by virtue of a *psi* sequence located at the 5' end of the viral genome.

The LTRs themselves are identical sequences that can be divided into three elements, which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both ends of the RNA and U5 is derived from the sequence unique to the 5' end of the RNA. The sizes of the three elements can vary considerably among different retroviruses.

In a defective retroviral vector genome *gag*, *pol* and *env* may be absent or not functional. The R regions at both ends of the RNA are repeated sequences. U5 and U3 represent unique sequences at the 5' and 3' ends of the RNA genome respectively.

In a typical retroviral vector for use in gene therapy, at least part of one or more of the *gag*, *pol* and *env* protein coding regions essential for replication may be removed from the virus. This makes the retroviral vector replication-defective. The removed portions may even be replaced by a nucleotide sequence of interest (NOI), such as a first nucleotide sequence of the invention, to generate a virus capable of integrating its genome into a host genome but wherein the modified viral genome is unable to propagate itself due to a lack of structural proteins. When integrated in the host genome, expression of the NOI occurs - resulting in,

for example, a therapeutic and/or a diagnostic effect. Thus, the transfer of an NOI into a site of interest is typically achieved by: integrating the NOI into the recombinant viral vector; packaging the modified viral vector into a virion coat; and allowing transduction of a site of interest - such as a targeted cell or a targeted cell population.

A minimal retroviral genome for use in the present invention will therefore comprise (5') R - U5 - one or more first nucleotide sequences - U3-R (3'). However, the plasmid vector used to produce the retroviral genome within a host cell/packaging cell will also include transcriptional regulatory control sequences operably linked to the retroviral genome to direct transcription of the genome in a host cell/packaging cell. These regulatory sequences may be the natural sequences associated with the transcribed retroviral sequence, i.e. the 5' U3 region, or they may be a heterologous promoter such as another viral promoter, for example the CMV promoter.

Some retroviral genomes require additional sequences for efficient virus production. For example, in the case of HIV, *rev* and RRE sequence are preferably included. However the requirement for *rev* and RRE can be reduced or eliminated by codon optimisation.

Once the retroviral vector genome is integrated into the genome of its target cell as proviral DNA, the ribozyme sequences need to be expressed. In a retrovirus, the promoter is located in the 5' LTR U3 region of the provirus. In retroviral vectors, the promoter driving expression of a therapeutic gene may be the native retroviral promoter in the 5' U3 region, or an alternative promoter engineered into the vector. The alternative promoter may physically replace the 5' U3 promoter native to the retrovirus, or it may be incorporated at a different place within the vector genome such as between the LTRs.

Thus, the first nucleotide sequence will also be operably linked to a transcriptional regulatory control sequence to allow transcription of the first nucleotide sequence to occur in the target cell. The control sequence will typically be active in mammalian cells. The control sequence may, for example, be a viral promoter such as the natural viral promoter or a CMV promoter or it may be a mammalian promoter. It is particularly preferred to use a promoter that is preferentially active in a particular cell type or tissue type in which the

virus to be treated primarily infects. Thus, in one embodiment, a tissue-specific regulatory sequences may be used. The regulatory control sequences driving expression of the one or more first nucleotide sequences may be constitutive or regulated promoters.

Replication-defective retroviral vectors are typically propagated, for example to prepare suitable titres of the retroviral vector for subsequent transduction, by using a combination of a packaging or helper cell line and the recombinant vector. That is to say, that the three packaging proteins can be provided *in trans*.

A "packaging cell line" contains one or more of the retroviral *gag, pol* and *env* genes. The packaging cell line produces the proteins required for packaging retroviral DNA but it cannot bring about encapsidation due to the lack of a *psi* region. However, when a recombinant vector carrying an NOI and a *psi* region is introduced into the packaging cell line, the helper proteins can package the *psi*-positive recombinant vector to produce the recombinant virus stock. This virus stock can be used to transduce cells to introduce the NOI into the genome of the target cells. It is preferred to use a *psi* packaging signal, called *psi* plus, that contains additional sequences spanning from upstream of the splice donor to downstream of the *gag* start codon (Bender *et al.* (46)) since this has been shown to increase viral titres.

The recombinant virus whose genome lacks all genes required to make viral proteins can tranduce only once and cannot propagate. These viral vectors which are only capable of a single round of transduction of target cells are known as replication defective vectors. Hence, the NOI is introduced into the host/target cell genome without the generation of potentially harmful retrovirus. A summary of the available packaging lines is presented in Coffin *et al.*, 1997 (*ibid*).

Retroviral packaging cell lines in which the *gag, pol* and *env* viral coding regions are carried on separate expression plasmids that are independently transfected into a packaging cell line are preferably used. This strategy, sometimes referred to as the three plasmid transfection method (Soneoka *et al.* (33)), reduces the potential for production of a replication-competent virus since three recombinant events are required for wild type viral

production. As recombination is greatly facilitated by homology, reducing or eliminating homology between the genomes of the vector and the helper can also be used to reduce the problem of replication-competent helper virus production.

An alternative to stably transfected packaging cell lines is to use transiently transfected cell lines. Transient transfections may advantageously be used to measure levels of vector production when vectors are being developed. In this regard, transient transfection avoids the longer time required to generate stable vector-producing cell lines and may also be used if the vector or retroviral packaging components are toxic to cells. Components typically used to generate retroviral vectors include a plasmid encoding the gag/pol proteins, a plasmid encoding the env protein and a plasmid containing an NOI. Vector production involves transient transfection of one or more of these components into cells containing the other required components. If the vector encodes toxic genes or genes that interfere with the replication of the host cell, such as inhibitors of the cell cycle or genes that induce apoptosis, it may be difficult to generate stable vector-producing cell lines, but transient transfection can be used to produce the vector before the cells die. Also, cell lines have been developed using transient transfection that produce vector titre levels that are comparable to the levels obtained from stable vector-producing cell lines (Pear *et al.* (47)).

Producer cells/packaging cells can be of any suitable cell type. Most commonly, mammalian producer cells are used but other cells, such as insect cells are not excluded. Clearly, the producer cells will need to be capable of efficiently translating the env and gag, pol mRNA. Many suitable producer/packaging cell lines are known in the art. The skilled person is also capable of making suitable packaging cell lines by, for example stably introducing a nucleotide construct encoding a packaging component into a cell line.

As will be discussed below, where the retroviral genome encodes an inhibitory RNA molecule capable of effecting the cleavage of *gag*, *pol* and/or *env* RNA transcripts, the nucleotide sequences present in the packaging cell line, either integrated or carried on plasmids, or in the transiently transfected producer cell line, which encode gag, pol and or env proteins will be modified so as to reduce or prevent binding of the inhibitory RNA molecule(s). In this way, the inhibitory RNA molecule(s) will not prevent expression of

components in packaging cell lines that are essential for packaging of viral particles.

It is highly desirable to use high-titre virus preparations in both experimental and practical applications. Techniques for increasing viral titre include using a *psi* plus packaging signal as discussed above and concentration of viral stocks. In addition, the use of different envelope proteins, such as the G protein from vesicular-stomatitis virus has improved titres following concentration to 10^9 per ml (Cosset *et al.* (48)). However, typically the envelope protein will be chosen such that the viral particle will preferentially infect cells that are infected with the virus which it desired to treat. For example where an HIV vector is being used to treat HIV infection, the env protein used will be the HIV env protein.

Suitable first nucleotide sequences for use according to the present invention encode gene products that result in the cleavage and/or enzymatic degradation of a target nucleotide sequence, which will generally be a ribonucleotide. As particular examples, ribozymes, and antisense sequences may be mentioned.

Ribozymes are RNA enzymes which cleave RNA at specific sites. Ribozymes can be engineered so as to be specific for any chosen sequence containing a ribozyme cleavage site. Thus, ribozymes can be engineered which have chosen recognition sites in transcribed viral sequences. By way of an example, ribozymes encoded by the first nucleotide sequence recognise and cleave essential elements of viral genomes required for the production of viral particles, such as packaging components. Thus, for retroviral genomes, such essential elements include the *gag*, *pol* and *env* gene products. A suitable ribozyme capable of recognising at least one of the gag, pol and env gene sequences, or more typically, the RNA sequences transcribed from these genes, is able to bind to and cleave such a sequence. This will reduce or prevent production of the gal, pol or env protein as appropriate and thus reduce or prevent the production of retroviral particles.

Ribozymes come in several forms, including hammerhead, hairpin and hepatitis delta antigenomic ribozymes. Preferred for use herein are hammerhead ribozymes, in part because of their relatively small size, because the sequence requirements for their target cleavage site are minimal and because they have been well characterised. The ribozymes

most commonly used in research at present are hammerhead and hairpin ribozymes.

Each individual ribozyme has a motif which recognises and binds to a recognition site in the target RNA. This motif takes the form of one or more "binding arms", generally two binding arms. The binding arms in hammerhead ribozymes are the flanking sequences Helix I and Helix III, which flank Helix II. These can be of variable length, usually between 6 to 10 nucleotides each, but can be shorter or longer. The length of the flanking sequences can affect the rate of cleavage. For example, it has been found that reducing the total number of nucleotides in the flanking sequences from 20 to 12 can increase the turnover rate of the ribozyme cleaving a HIV sequence, by 10-fold (44). A catalytic motif in the ribozyme Helix II in hammerhead ribozymes cleaves the target RNA at a site which is referred to as the cleavage site. Whether or not a ribozyme will cleave any given RNA is determined by the presence or absence of a recognition site for the ribozyme containing an appropriate cleavage site.

Each type of ribozyme recognises its own cleavage site. The hammerhead ribozyme cleavage site has the nucleotide base triplet GUX directly upstream where G is guanine, U is uracil and X is any nucleotide base. Hairpin ribozymes have a cleavage site of BCUGNYR, where B is any nucleotide base other than adenine, N is any nucleotide, Y is cytosine or thymine and R is guanine or adenine. Cleavage by hairpin ribozymes takes places between the G and the N in the cleavage site.

The nucleic acid sequences encoding the packaging components (the "third nucleotide sequences") may be resistant to the ribozyme or ribozymes because they lack any cleavage sites for the ribozyme or ribozymes. This prohibits enzymatic activity by the ribozyme or ribozymes and therefore there is no effective recognition site for the ribozyme or ribozymes. Alternatively or additionally, the potential recognition sites may be altered in the flanking sequences which form the part of the recognition site to which the ribozyme binds. This either eliminates binding of the ribozyme motif to the recognition site, or reduces binding capability enough to destabilise any ribozyme-target complex and thus reduce the specificity and catalytic activity of the ribozyme. Where the flanking sequences only are altered, they are preferably altered such that catalytic activity of the ribozyme at

the altered target sequence is negligible and is effectively eliminated.

Preferably, a series of several anti-HIV ribozymes is employed in the invention (5, 7, 10, 13, 21, 36, 38, 40). These can be any anti-HIV ribozymes but must include one or more which cleave the RNA that is required for the expression of *gag*, *pol* or *env*. Preferably, a plurality of ribozymes is employed, together capable of cleaving *gag, pol* and *env* RNA of the native retrovirus at a plurality of sites. Since HIV exists as a population of quasispecies, not all of the target sequences for the ribozymes will be included in all HIV variants. The problem presented by this variability can be overcome by using multiple ribozymes. Multiple ribozymes can be included in series in a single vector and can function independently when expressed as a single RNA sequence. A single RNA containing two or more ribozymes having different target recognition sites may be referred to as a multitarget ribozyme. The placement of ribozymes in series has been demonstrated to enhance cleavage. The use of a plurality of ribozymes is not limited to treating HIV infection but may be used in relation to other viruses, retroviruses or otherwise.

Antisense technology is well known on the art. There are various mechanisms by which antisense sequences are believed to inhibit gene expression. One mechanism by which antisense sequences are believed to function is the recruitment of the cellular protein RNAseH to the target sequence/antisense construct heteroduplex which results in cleavage and degradation of the heteroduplex. Thus the antisense construct, by contrast to ribozymes, can be said to lead indirectly to cleavage/degradation of the target sequence. Thus according to the present invention, a first nucleotide sequence may encode an antisense RNA that binds to either a gene encoding an essential/packaging component or the RNA transcribed from said gene such that expression of the gene is inhibited, for example as a result of RNAseH degradation of a resulting heteroduplex. It is not necessary for the antisense construct to encode the entire complementary sequence of the gene encoding an essential/packaging component - a portion may suffice. The skilled person will easily be able to determine how to design a suitable antisense construct.

By contrast, the nucleic acid sequences encoding the essential/packaging components of the viral particles required for the assembly of viral particles in the host cells/producer

cells/packaging cells (the third nucleotide sequences) are resistant to the inhibitory RNA molecules encoded by the first nucleotide sequence. For example in the case of ribozymes, resistance is typically by virtue of alterations in the sequences which eliminate the ribozyme recognition sites. At the same time, the amino acid coding sequence for the essential/packaging components is retained so that the viral components encoded by the sequences remain the same, or at least sufficiently similar that the function of the essential/packaging components is not compromised.

The term “viral polypeptide required for the assembly of viral particles” means a polypeptide normally encoded by the viral genome to be packaged into viral particles, in the absence of which the viral genome cannot be packaged. For example, in the context of retroviruses such polypeptides would include gag, pol and env. The terms “packaging component” and “essential component” are also included within this definition.

In the case of antisense sequences, the third nucleotide sequence differs from the second nucleotide sequence encoding the target viral packaging component antisense sequence to the extent that although the antisense sequence can bind to the second nucleotide sequence, or transcript thereof, the antisense sequence can not bind effectively to the third nucleotide sequence or RNA transcribed from therefrom. The changes between the second and third nucleotide sequences will typically be conservative changes, although a small number of amino acid changes may be tolerated provided that, as described above, the function of the essential/packaging components is not significantly impaired.

Preferably, in addition to eliminating the ribozyme recognition sites, the alterations to the coding sequences for the viral components improve the sequences for codon usage in the mammalian cells or other cells which are to act as the producer cells for retroviral vector particle production. This improvement in codon usage is referred to as “codon optimisation”. Many viruses, including HIV and other lentiviruses, use a large number of rare codons and by changing these to correspond to commonly used mammalian codons, increased expression of the packaging components in mammalian producer cells can be achieved. Codon usage tables are known in the art for mammalian cells, as well as for a variety of other organisms.

Thus preferably, the sequences encoding the packaging components are codon optimised. More preferably, the sequences are codon optimised in their entirety. Following codon optimisation, it is found that there are numerous sites in the wild type *gag, pol* and *env* sequences which can serve as ribozyme recognition sites and which are no longer present in the sequences encoding the packaging components. In an alternative but less practical strategy, the sequences encoding the packaging components can be altered by targeted conservative alterations so as to render them resistant to selected ribozymes capable of cleaving the wild type sequences.

An additional advantage of codon optimising HIV packaging components is that this can increase gene expression. In particular, it can render *gag, pol* expression Rev independent so that *rev* and RRE need not be included in the genome (11). Rev-independent vectors are therefore possible. This in turn enables the use of anti-*rev* or RRE factors in the retroviral vector.

As described above, the packaging components for a retroviral vector include expression products of *gag, pol* and *env* genes. In accordance with the present invention, *gag* and *pol* employed in the packaging system are derived from the target retrovirus on which the vector genome is based. Thus, in the RNA transcript form, *gag* and *pol* would normally be cleavable by the ribozymes present in the vector genome. The *env* gene employed in the packaging system may be derived from a different virus, including other retroviruses such as MLV and non-retroviruses such as VSV (a Rhabdovirus), in which case it may not need any sequence alteration to render it resistant to ribozyme cleavage. Alternatively, *env* may be derived from the same retrovirus as *gag* and *pol*, in which case any recognition sites for the ribozymes will need to be eliminated by sequence alteration.

The process of producing a retroviral vector in which the envelope protein is not the native envelope of the retrovirus is known as "pseudotyping". Certain envelope proteins, such as MLV envelope protein and vesicular stomatitis virus G (VSV-G) protein, pseudotype retroviruses very well. Pseudotyping can be useful for altering the target cell range of the retrovirus. Alternatively, to maintain target cell specificity for target cells infected with the

particular virus it is desired to treat, the envelope protein may be the same as that of the target virus, for example HIV.

Other therapeutic coding sequences may be present along with the first nucleotide sequence or sequences. Other therapeutic coding sequences include, but are not limited to, sequences encoding cytokines, hormones, antibodies, immunoglobulin fusion proteins, enzymes, immune co-stimulatory molecules, anti-sense RNA, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen, a single chain antibody, tumour suppresser protein and growth factors. When included, such coding sequences are operatively linked to a suitable promoter, which may be the promoter driving expression of the first nucleotide sequence or a different promoter or promoters.

Thus the invention comprises two components. The first is a genome construction that will be packaged by viral packaging components and which carries a series of anti-viral inhibitory RNA molecules such as anti-HIV ribozymes (5, 7, 10, 13, 21, 36, 38, 40). These could be any anti-HIV ribozymes but the key issue for this invention is that some of them cleave RNA that is required for the expression of native or wild type HIV *gag*, *pol* or *env* coding sequences. The second component is the packaging system which comprises a cassette for the expression of HIV gag, pol and a cassette either for HIV env or an envelope gene encoding a pseudotyping envelope protein - the packaging system beig resistant to the inhibitory RNA molecules.

The viral particles of the present invention, and the viral vector system and methods used to produce may thus be used to treat or prevent viral infections, preferably retroviral infections, in particular lentiviral, especially HIV, infections. Specifically, the viral particles of the invention, typically produced using the viral vector system of the present invention may be used to deliver inhibitory RNA molecules to a human or animal in need of treatment for a viral infection.

Alternatively, or in addition, the viral production system may be used to transfect cells obtained from a patient *ex vivo* and then returned to the patient. Patient cells transfected ex vivo may be formulated as a pharmaceutical composition (see below) prior to

readminstration to the patient.

Preferably the viral particles are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Thus, the present invention also provides a pharmaceutical composition for treating an individual, wherein the composition comprises a therapeutically effective amount of the viral particle of the present invention, together with a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. The pharmaceutical composition may be for human or animal usage.

The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s), and other carrier agents that may aid or increase the viral entry into the target site (such as for example a lipid delivery system).

The pharmaceutical composition may be formulated for parenteral, intramuscular, intravenous, intracranial, subcutaneous, intraocular or transdermal administration.

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

The amount of virus administered is typically in the range of from 10^3 to 10^{10} pfu, preferably from 10^5 to 10^8 pfu, more preferably from 10^6 to 10^7 pfu. When injected, typically 1-10 μl of virus in a pharmaceutically acceptable suitable carrier or diluent is administered.

When the polynucleotide/vector is administered as a naked nucleic acid, the amount of nucleic acid administered is typically in the range of from 1 μg to 10 mg, preferably from 100 μg to 1 mg.

Where the first nucleotide sequence (or other therapeutic sequence) is under the control of an inducible regulatory sequence, it may only be necessary to induce gene expression for the duration of the treatment. Once the condition has been treated, the inducer is removed and expression of the NOI is stopped. This will clearly have clinical advantages. Such a system may, for example, involve administering the antibiotic tetracycline, to activate gene expression via its effect on the tet repressor/VP16 fusion protein.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention. The Examples refer to the Figures. In the Figures:

Figure 1 shows schematically ribozymes inserted into four different HIV vectors;

Figure 2 shows schematically how to create a suitable 3' LTR by PCR;

Figure 3 shows the codon usage table for wild type HIV *gag,pol* of strain HXB2 (accession number: K03455).

Figure 4 shows the codon usage table of the codon optimised sequence designated gag,pol-SYNgp.

Figure 5 shows the codon usage table of the wild type HIV *env* called env-mn.

Figure 6 shows the codon usage table of the codon optimised sequence of HIV *env* designated SYNgp160mn.

Figure 7 shows three plasmid constructs for use in the invention.

Figure 8 shows the principle behind two systems for producing retroviral vector particles.

The invention will now be further described in the Examples which follow, which are intended as an illustration only and do not limit the scope of the invention.

EXAMPLES

Example 1 - Construction of a Genome

The HIV *gag.pol* sequence was codon optimised (Figure 4 and SEQ I.D. No. 1) and synthesised using overlapping oligos of around 40 nucleotides. This has three advantages. Firstly it allows an HIV based vector to carry ribozymes and other therapeutic factors. Secondly the codon optimisation generates a higher vector titre due to a higher level of gene expression. Thirdly *gag.pol* expression becomes *rev* independent which allows the use of anti-*rev* or RRE factors.

Conserved sequences within *gag.pol* were identified by reference to the HIV Sequence database at Los Alamos National Laboratory (http:// hiv-web.lanl.gov/) and used to design ribozymes. Because of the variability between subtypes of HIV-1 the ribozymes were designed to cleave the predominant subtype within North America, Latin America and the Caribbean, Europe, Japan and Australia; that is subtype B. The sites chosen were cross-referenced with the synthetic *gagpol* sequence to ensure that there was a low possibility of cutting the codon optimised gagpol mRNA. The ribozymes were designed with *Xho*I and

*Sal*I sites at the 5' and 3' end respectively. This allows the construction of separate and tandem ribozymes.

The ribozymes are hammerhead (25) structures of the following general structure:

Helix I	Helix II	Helix III
5'-NNNNNNNN~	CUGAUGAGGCCGAAAGGCCGAA	~NNNNNNNN~

The catalytic domain of the ribozyme (Helix II) can tolerate some changes without reducing catalytic turnover.

The cleavage sites, targeting *gag* and *pol*, with the essential GUX triplet (where X is any nucleotide base) are as follows:

GAG 1	5'	UAGUAAGAAUGUAUAGCCCUAC
GAG 2	5'	AACCCAGAUUGUAAGACUAUUU
GAG 3	5'	UGUUUCAAUUGUGGCAAAGAAG
GAG 4	5'	AAAAAGGGCUGUUGGAAAUGUG
POL 1	5'	ACGACCCCUCGUCACAAUAAAG
POL 2	5'	GGAAUUGGAGGUUUUAUCAAAG
POL 3	5'	AUAUUUUUCAGUUCCCUUAGAU
POL 4	5'	UGGAUGAUUUGUAUGUAGGAUC
POL 5	5'	CUUUGGAUGGGUUAUGAACUCC
POL 6	5'	CAGCUGGACUGUCAAUGACAUA
POL 7	5'	AACUUUCUAUGUAGAUGGGGCA
POL 8	5'	AAGGCCGCCUGUUGGUGGGCAG
POL 9	5'	UAAGACAGCAGUACAAAUGGCA

The ribozymes are inserted into four different HIV vectors (pH4 (10), pH6, pH4.1, or pH6.1) (Figure 1). In pH4 and pH6, transcription of the ribozymes is driven by an internal HCMV promoter (9). From pH4.1 and pH6.1, the ribozymes are expressed from the 5' LTR. The major difference between pH4 and pH6 (and pH4.1 and pH6.1) resides in the 3'

LTR in the production plasmid. pH4 and pH4.1 have the HIV U3 in the 3' LTR. pH6 and pH6.1 have HCMV in the 3'LTR. The HCMV promoter replaces most of the U3 and will drive expression at high constitutive levels while the HIV-1 U3 will support a high level of expression only in the presence of Tat.

The HCMV/HIV-1 hybrid 3' LTR is created by recombinant PCR with three PCR primers (Figure 2). The first round of PCR is performed with RIB1 and RIB2 using pH4 (12) as the template to amplify the HIV-1 HXB2 sequence 8900-9123. The second round of PCR makes the junction between the 5' end of the HIV-1 U3 and the HCMV promoter by amplifying the hybrid 5' LTR from pH4. The PCR product from the first PCR reaction and RIB3 serves as the 5' primer and 3' primer respectively.

```
RIB1:   5'-CAGCTGCTCGAGCAGCTGAAGCTTGCATGC-3'
RIB2:   5'-GTAAGTTATGTAACGGACGATATCTTGTCTTCTT-3'
RIB3:   5'-CGCATAGTCGACGGGCCCGCCACTGCTAGAGATTTTC-3'
```

The PCR product is then cut with *Sph*I and *Sal*I and inserted into pH4 thereby replacing the 3' LTR. The resulting plasmid is designated pH6. To construct pH4.1 and pH6.1, the internal HCMV promoter *(Spe*I - *Xho*I*)* in pH4 and pH6 is replaced with the polycloning site of pBluescript II KS+ (Stratagene) *(Spe*I - *Xho*I*)*.

The ribozymes are inserted into the *Xho*I sites in the genome vector backbones. Any ribozymes in any configuration could be used in a similar way.

Example 2 - Construction of a Packaging System

The packaging system can take various forms. In a first form of packaging system, the HIV gag, pol components are co-expressed with the HIV env coding sequence. In this case, both the gag, pol and the env coding sequences are altered such that they are resistant to the anti-HIV ribozymes that are built into the genome. At the same time as altering the codon usage to achieve resistance, the codons can be chosen to match the usage pattern of the most highly expressed mammalian genes. This dramatically increases expression

levels (28, 29) and so increases titre. A codon optimised HIV env coding sequence has been described by Haas *et al* (9). In the present example, a modified codon optimised HIV env sequence is used (SEQ I.D. No. 3). The corresponding env expression plasmid is designated pSYNgp160mn. The modified sequence contains extra motifs not used by Haas *et al*. The extra sequences were taken from the HIV env sequence of strain MN and codon optimised. Any similar modification of the nucleic acid sequence would function similarly as long as it used codons corresponding to abundant tRNAs (42) and lead to resistance to the ribozymes in the genome.

In one example of a gag, pol coding sequence with optimised codon usage, overlapping oligonucleotides are synthesised and then ligated together to produce the synthetic coding sequence. The sequence of a wild-type (Genbank accession no. K03455) and synthetic (gagpol-SYNgp) gagpol sequence is shown in SEQ I.D. Nos 1 and 2, respectively and their codon usage is shown in Figures 3 and 4, respectively. The sequence of a wild type env coding sequence (Genbank Accession No. M17449) is given in SEQ I.D. No 3, the sequence of a synthetic codon optimised sequence is given in SEQ. I.D. No. 4 and their codon usage tables are given in Figures 5 and 6, respectively. As with the env coding sequence any gag, pol sequence that achieves resistance to the ribozymes could be used. The synthetic sequence shown is designated gag, pol-SYNgp and has an *EcoR*I site at the 5' end and a *Not*I site at the 3' end. It is inserted into pClneo (Promega) to produce plasmid pSYNgp.

In a second form of the packaging system a synthetic gag, pol cassette is coexpressed with a non-HIV envelope coding sequence that produces a surface protein that pseudotypes HIV. This could be for example VSV-G (20, 41), amphotropic MLV env (6, 34) or any other protein that would be incorporated into the HIV particle (37). This includes molecules capable of targeting the vector to specific tissues. Coding sequences for non-HIV envelope proteins not cleaved by the ribozymes and so no sequence modification is required (although some sequence modification may be desirable for other reasons such as optimisation for codon usage in mammalian cells).

Example 3 - Vector Particle Production

Vector particles can be produced either from a transient three-plasmid transfection system similar to that described by Soneoka *et al.* (33) or from producer cell lines similar to those used for other retroviral vectors (20, 35, 39). These principles are illustrated in Figures 7 and 8. For example, by using pH6Rz, pSYNgp and pRV67 (VSV-G expression plasmid) in a three plasmid transfection of 293T cells (Figure 8), as described by Soneoka *et al* (33), vector particles designated H6Rz-VSV are produced. These transduce the H6Rz genome to CD4+ cells such as C1866 or Jurkat and produce the multitarget ribozymes. HIV replication in these cells is now severely restricted.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

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CLAIMS

1. A viral vector system comprising:

(i) a first nucleotide sequence encoding a gene product capable of binding to and effecting the cleavage, directly or indirectly, of a second nucleotide sequence, or transcription product thereof, encoding a viral polypeptide required for the assembly of viral particles; and

(ii) a third nucleotide sequence encoding said viral polypeptide required for the assembly of viral particles, which third nucleotide sequence has a different nucleotide sequence to the second nucleotide sequence such that the third nucleotide sequence, or transcription product thereof, is resistant to cleavage directed by said gene product.

2. A viral vector production system comprising:

(i) a viral genome comprising at least one first nucleotide sequence encoding a gene product capable of binding to and effecting the cleavage, directly or indirectly, of a second nucleotide sequence, or transcription product thereof, encoding a viral polypeptide required for the assembly of viral particles;

(ii) a third nucleotide sequence encoding said viral polypeptide required for the assembly of the viral genome into viral particles, which third nucleotide sequence has a different nucleotide sequence to the second nucleotide sequence such that said third nucleotide sequence, or transcription product thereof, is resistant to cleavage directed by said gene product.

3. A system according to claim 1 or 2 wherein the gene product is selected from a ribozyme and an anti-sense ribonucleic acid.

4. A system according to any one of claims 1 to 3 wherein the viral vector is a retroviral vector.

5. A system according to claim 4 wherein the retroviral vector is a lentiviral vector.

6. A system according to claim 5 wherein the lentiviral vector is an HIV vector.

7. A system according to any one of claims 4 to 6 wherein the polypeptide required for the assembly of viral particles is selected from gag, pol and env proteins.

8. A system according to claim 7 wherein at least the gag and pol proteins are from a lentivirus.

9. A system according to claim 7 wherein the env protein is from a lentivirus.

10. A system according to claim 8 or 9 wherein the lentivirus is HIV.

11. A system according to any one of the preceding claims wherein the third nucleotide sequence is resistant to cleavage directed by the gene product as a result of one or more conservative alterations in the nucleotide sequence which remove cleavage sites recognised by the at least one gene product and/or binding sites for the at least one gene product

12. A system according to any one of claims 1 to 10 wherein the third nucleotide sequence is adapted to be resistant to cleavage by the at least one gene product.

13. A system according to any one of the preceding claims wherein the third nucleotide sequence is codon optimised for expression in producer cells.

14. A system according to claim 13, wherein the producer cells are mammalian cells.

15. A system according to any one of the preceding claims comprising a plurality of first nucleotide sequences and third nucleotide sequences as defined therein.

16. A viral particle comprising a viral vector genome as defined in any one of claims 2 to 15 and one or more third nucleotide sequences as defined in any of claims 2 to 15.

17. A viral particle produced using a viral vector production system according to any one of claims 2 to 15.

18. A method for producing a viral particle which method comprises introducing into a host cell (i) a viral genome as defined in any one of claims 2 to 15 (ii) one or more third nucleotide sequences as defined in any of claims 2 to 15 and (iii) nucleotide sequences encoding the other essential viral packaging components not encoded by the one or more third nucleotide sequences.

19. A viral particle produced by the method of claim 18.

20. A pharmaceutical composition comprising a viral particle according to claims 16, 17 or 19 together with a pharmaceutically acceptable carrier or diluent.

21. A viral system according to any one of claims 1 to 16 or a viral particle according to claims 16, 17 or 19 in treating a viral infection.

22. A viral system according to any one of claims 1 to 16 for use in a method of producing viral particles.

Figure 1

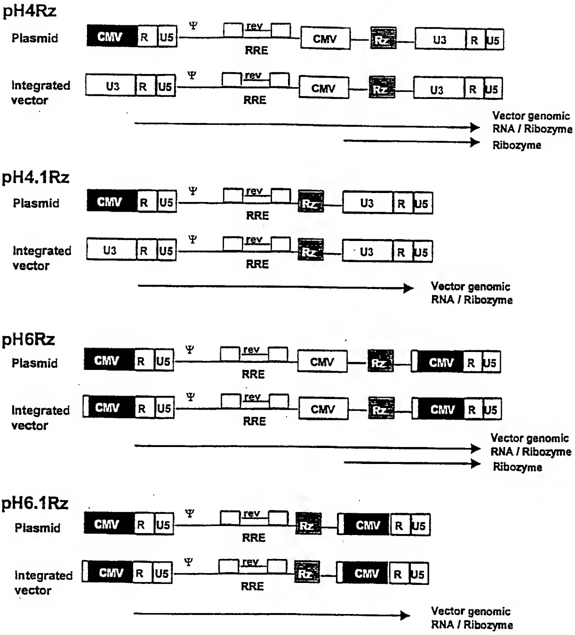
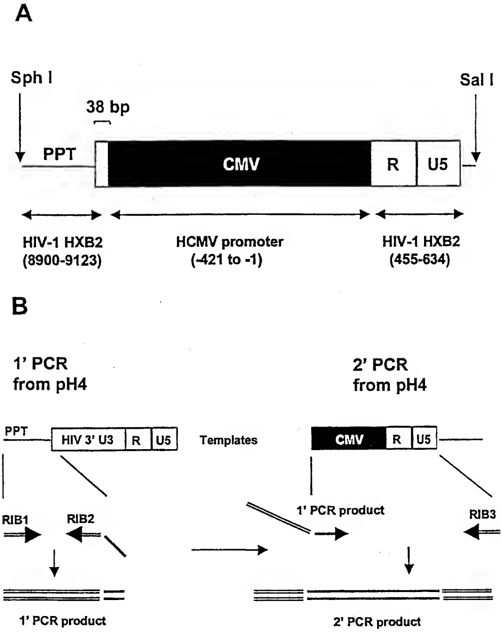

pH4Rz
Plasmid
CMV
R
U5
Ψ
rev
RRE
CMV
Rz
U3
R
U5
Integrated
vector
U3
R
U5
Vector genomic
RNA / Ribozyme
Ribozyme
pH4.1Rz
Plasmid
Integrated
vector
Vector genomic
RNA / Ribozyme
pH6Rz
Plasmid
Integrated
vector
Vector genomic
RNA / Ribozyme
Ribozyme
pH6.1Rz
Plasmid
Integrated
vector
Vector genomic
RNA / Ribozyme

figure 2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Sph I
Sal I
38 bp
PPT
CMV
R
U5
HIV-1 HXB2
(8900-9123)
HCMV promoter
(-421 to -1)
HIV-1 HXB2
(455-634)
B
1' PCR
from pH4
2' PCR
from pH4
PPT
HIV 3' U3
R
U5
Templates
CMV
R
U5
1' PCR product
RIB1
RIB2
RIB3
1' PCR product
2' PCR product

Figure 3

gagpol-HXB2 -> Codon Usage

DNA sequence 4308 b.p. ATGGGTGCGAGA ... GATGAGGATTAG linear

1436 codons

MW : 161929 Dalton CAI(S.c.) : 0.083 CAI(E.c.) : 0.151

TTT	phe F	21	TCT	ser S	3	TAT	tyr Y	30	TGT	cys C	18
TTC	phe F	14	TCC	ser S	3	TAC	tyr Y	9	TGC	cys C	2
TTA	leu L	46	TCA	ser S	19	TAA	OCH Z	-	TGA	OPA Z	-
TTG	leu L	11	TCG	ser S	1	TAG	AMB Z	1	TGG	trp W	37
CTT	leu L	13	CCT	pro P	21	CAT	his H	20	CGT	arg R	-
CTC	leu L	7	CCC	pro P	14	CAC	his H	7	CGC	arg R	-
CTA	leu L	17	CCA	pro P	41	CAA	gln Q	56	CGA	arg R	3
CTG	leu L	16	CCG	pro P	-	CAG	gln Q	39	CGG	arg R	3
ATT	ile I	30	ACT	thr T	24	AAT	asn N	42	AGT	ser S	18
ATC	ile I	14	ACC	thr T	20	AAC	asn N	16	AGC	ser S	16
ATA	ile I	56	ACA	thr T	43	AAA	lys K	88	AGA	arg R	45
ATG	met M	29	ACG	thr T	1	AAG	lys K	34	AGG	arg R	18
GTT	val V	15	GCT	ala A	17	GAT	asp D	37	GGT	gly G	11
GTC	val V	11	GCC	ala A	19	GAC	asp D	26	GGC	gly G	10
GTA	val V	55	GCA	ala A	55	GAA	glu E	75	GGA	gly G	61
GTG	val V	15	GCG	ala A	5	GAG	glu E	32	GGG	gly G	26

Figure 4

gagpol-SYNgp [1 to 4308] -> Codon Usage

DNA sequence 4308 b.p. ATGGGCGCCCGC ... GATGAGGATTAG linear

1436 codons

MW : 161929 Dalton CAI(S.c.) : 0.080 CAI(E.c.) : 0.296

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TTC phe F	30	TCC ser S	11	TAC tyr Y	29	TGC cys C	14
TTA leu L	2	TCA ser S	4	TAA OCH Z	-	TGA OPA Z	-
TTG leu L	7	TCG ser S	6	TAG AMB Z	1	TGG trp W	37
CTT leu L	3	CCT pro P	14	CAT his H	6	CGT arg R	2
CTC leu L	22	CCC pro P	39	CAC his H	21	CGC arg R	34
CTA leu L	6	CCA pro P	10	CAA gln Q	14	CGA arg R	3
CTG leu L	70	CCG pro P	13	CAG gln Q	81	CGG arg R	10
ATT ile I	17	ACT thr T	11	AAT asn N	13	AGT ser S	7
ATC ile I	79	ACC thr T	48	AAC asn N	45	AGC ser S	27
ATA ile I	4	ACA thr T	13	AAA lys K	25	AGA arg R	7
ATG met M	29	ACG thr T	16	AAG lys K	97	AGG arg R	13
GTT val V	5	GCT ala A	15	GAT asp D	19	GGT gly G	10
GTC val V	27	GCC ala A	56	GAC asp D	44	GGC gly G	54
GTA val V	6	GCA ala A	13	GAA glu E	29	GGA gly G	16
GTG val V	58	GCG ala A	12	GAG glu E	78	GGG gly G	28

Figure 5

env-mn [1 to 2571] -> Codon Usage

DNA sequence 2571 b.p. ATGAGAGTGAAG ... GCTTTGCTATAA linear

857 codons

MW : 97078 Dalton CAI(S.c.) : 0.083 CAI(E.c.) : 0.140

TTT phe F	13	TCT ser S	7	TAT tyr Y	15	TGT cys C	16
TTC phe F	11	TCC ser S	3	TAC tyr Y	7	TGC cys C	5
TTA leu L	20	TCA ser S	13	TAA OCH Z	1	TGA OPA Z	-
TTG leu L	17	TCG ser S	2	TAG AMB Z	-	TGG trp W	30
CTT leu L	9	CCT pro P	5	CAT his H	8	CGT arg R	-
CTC leu L	11	CCC pro P	9	CAC his H	6	CGC arg R	2
CTA leu L	12	CCA pro P	12	CAA gln Q	22	CGA arg R	1
CTG leu L	15	CCG pro P	2	CAG gln Q	19	CGG arg R	1
ATT ile I	21	ACT thr T	16	AAT asn N	50	AGT ser S	18
ATC ile I	10	ACC thr T	14	AAC asn N	13	AGC ser S	11
ATA ile I	32	ACA thr T	28	AAA lys K	32	AGA arg R	30
ATG met M	17	ACG thr T	5	AAG lys K	14	AGG arg R	15
GTT val V	8	GCT ala A	16	GAT asp D	18	GGT gly G	10
GTC val V	9	GCC ala A	7	GAC asp D	14	GGC gly G	6
GTA val V	26	GCA ala A	20	GAA glu E	36	GGA gly G	28
GTG val V	12	GCG ala A	5	GAG glu E	10	GGG gly G	12

Figure 6

SYNgp160mn -> Codon Usage

DNA sequence 2571 b.p. ATGAGGGTGAAG ... GCGCTGCTGTAA linear

857 codons

MW : 97078 Dalton CAI(S.c.) : 0.074 CAI(E.c.) : 0.419

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TTC phe F	24	TCC ser S	4	TAC tyr Y	21	TGC cys C	21
TTA leu L	-	TCA ser S	-	TAA OCH Z	1	TGA OPA Z	-
TTG leu L	-	TCG ser S	-	TAG AMB Z	-	TGG trp W	30
CTT leu L	-	CCT pro P	-	CAT his H	2	CGT arg R	1
CTC leu L	20	CCC pro P	26	CAC his H	12	CGC arg R	36
CTA leu L	1	CCA pro P	-	CAA gln Q	-	CGA arg R	-
CTG leu L	63	CCG pro P	2	CAG gln Q	41	CGG arg R	4
ATT ile I	2	ACT thr T	-	AAT asn N	2	AGT ser S	-
ATC ile I	61	ACC thr T	59	AAC asn N	61	AGC ser S	48
ATA ile I	-	ACA thr T	-	AAA lys K	1	AGA arg R	2
ATG met M	17	ACG thr T	4	AAG lys K	45	AGG arg R	6
GTT val V	-	GCT ala A	-	GAT asp D	2	GGT gly G	1
GTC val V	1	GCC ala A	40	GAC asp D	30	GGC gly G	47
GTA val V	1	GCA ala A	-	GAA glu E	3	GGA gly G	-
GTG val V	53	GCG ala A	8	GAG glu E	43	GGG gly G	8

Figure 7

HIV Constructs

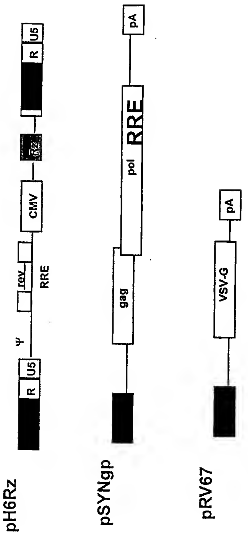
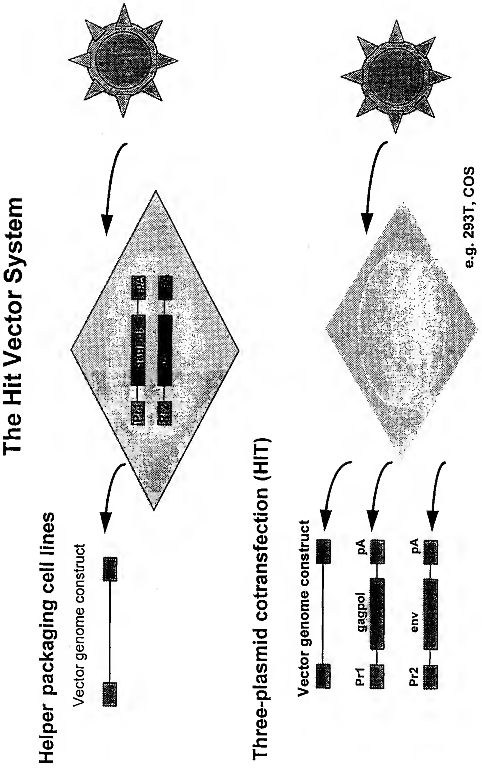


Figure 8


The Hit Vector System
Helper packaging cell lines
Vector genome construct
Three-plasmid cotransfection (HIT)
Vector genome construct
Pr1
gagpol
pA
Pr2
env
pA
e.g. 293T, COS

SEQUENCE LISTING PART OF THE DESCRIPTION

SEQ. ID. NO. 1 - Wild type gagpol sequence for strain HXB2 (accession no. K03455)

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CTAGAACGAT TCGCAGTTAA TCCTGGCCTG TTAGAAACAT CAGAAGGCTG TAGACAAATA 180
CTGGGACAGC TACAACCATC CCTTCAGACA GGATCAGAAG AACTTAGATC ATTATATAAT 240
ACAGTAGCAA CCCTCTATTG TGTGCATCAA AGGATAGAGA TAAAAGACAC CAAGGAAGCT 300
TTAGACAAGA TAGAGGAAGA GCAAAACAAA AGTAAGAAAA AAGCACAGCA AGCAGCAGCT 360
GACACAGGAC ACAGCAATCA GGTCAGCCAA AATTACCCTA TAGTGCAGAA CATCCAGGGG 420
CAAATGGTAC ATCAGGCCAT ATCACCTAGA ACTTTAAATG CATGGGTAAA AGTAGTAGAA 480
GAGAAGGCTT TCAGCCCAGA AGTGATACCC ATGTTTTCAG CATTATCAGA AGGAGCCACC 540
CCACAAGATT TAAACACCAT GCTAAACACA GTGGGGGGAC ATCAAGCAGC CATGCAAATG 600
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GGGCCTATTG CACCAGGCCA GATGAGAGAA CCAAGGGGAA GTGACATAGC AGGAACTACT 720
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SEQ. ID. NO. 3 - Envelope Gene from HIV-1 MN (Genbank accession no. M17449)

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TGTACAAGAC CCAACTACAA TAAAAGAAAA AGGATACATA TAGGACCAGG GAGAGCATTT 960
TATACAACAA AAAATATAAT AGGAACTATA AGACAAGCAC ATTGTAACAT TAGTAGAGCA 1020
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ACAATAGTCT TTAATCAATC CTCAGGAGGG GACCCAGAAA TTGTAATGCA CAGTTTTAAT 1140
TGTGGAGGGG AATTTTTCTA CTGTAATACA TCACCACTGT TTAATAGTAC TTGGAATGGT 1200
AATAATACTT GGAATAATAC TACAGGGTCA AATAACAATA TCACACTTCA ATGCAAAATA 1260
AAACAAATTA TAAACATGTG GCAGGAAGTA GGAAAAGCAA TGTATGCCCC TCCCATTGAA 1320
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GACACGGACA CGAACGACAC CGAGATCTTC AGACCTGGAG GAGGAGATAT GAGGGACAAT 1440
TGGAGAAGTG AATTATATAA ATATAAAGTA GTAACAATTG AACCATTAGG AGTAGCACCC 1500
ACCAAGGCAA AGAGAAGAGT GGTGCAGAGA GAAAAAAGAG CAGCGATAGG AGCTCTGTTC 1560
CTTGGGTTCT TAGGAGCAGC AGGAAGCACT ATGGGCGCAG CGTCAGTGAC GCTGACGGTA 1620
CAGGCCAGAC TATTATTGTC TGGTATAGTG CAACAGCAGA ACAATTTGCT GAGGGCCATT 1680
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GGAAAACTCA TTTGCACCAC TACTGTGCCT TGGAATGCTA GTTGGAGTAA TAAATCTCTG 1860
GATGATATTT GGAATAACAT GACCTGGATG CAGTGGGAAA GAGAAATTGA CAATTACACA 1920
AGCTTAATAT ACTCATTACT AGAAAAATCG CAAACCCAAC AAGAAAAGAA TGAACAAGAA 1980
TTATTGGAAT TGGATAAATG GGCAAGTTTG TGGAATTGGT TTGACATAAC AAATTGGCTG 2040
TGGTATATAA AAATATTCAT AATGATAGTA GGAGGCTTGG TAGGTTTAAG AATAGTTTTT 2100

GCTGTACTTT CTATAGTGAA TAGAGTTAGG CAGGGATACT CACCATTGTC GTTGCAGACC 2160
CGCCCCCCAG TTCCGAGGGG ACCCGACAGG CCCGAAGGAA TCGAAGAAGA AGGTGGAGAG 2220
AGAGACAGAG ACACATCCGG TCGATTAGTG CATGGATTCT TAGCAATTAT CTGGGTCGAC 2280
CTGCGGAGCC TGTTCCTCTT CAGCTACCAC CACAGAGACT TACTCTTGAT TGCAGCGAGG 2340
ATTGTGGAAC TTCTGGGACG CAGGGGGTGG GAAGTCCTCA AATATTGGTG GAATCTCCTA 2400
CAGTATTGGA GTCAGGAACT AAAGAGTAGT GCTGTTAGCT TGCTTAATGC CACAGCTATA 2460
GCAGTAGCTG AGGGGACAGA TAGGGTTATA GAAGTACTGC AAAGAGCTGG TAGAGCTATT 2520
CTCCACATAC CTACAAGAAT AAGACAGGGC TTGGAAAGGG CTTTGCTATA A 2571

SEQ. I.D. NO. 4 - SYNgp-160mn - codon optimised env sequence

ATGAGGGTGA AGGGGATCCG CCGCAACTAC CAGCACTGGT GGGGCTGGGG CACGATGCTC 60
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GTGCCCGTGT GGAAGGAGGC CACCACCACC CTGTTCTGCG CCAGCGACGC CAAGGCGTAC 180
GACACCGAGG TGCACAACGT GTGGGCCACC CAGGCGTGCG TGCCCACCGA CCCCAACCCC 240
CAGGAGGTGG AGCTCGTGAA CGTGACCGAG AACTTCAACA TGTGGAAGAA CAACATGGTG 300
GAGCAGATGC ATGAGGACAT CATCAGCCTG TGGGACCAGA GCCTGAAGCC CTGCGTGAAG 360
CTGACCCCCC TGTGCGTGAC CCTGAACTGC ACCGACCTGA GGAACACCAC CAACACCAAC 420
AACAGCACCG CCAACAACAA CAGCAACAGC GAGGGCACCA TCAAGGGCGG CGAGATGAAG 480
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CTGTACAAGC TGGATATCGT GAGCATCGAC AACGACAGCA CCAGCTACCG CCTGATCTCC 600
TGCAACACCA GCGTGATCAC CCAGGCCTGC CCCAAGATCA GCTTCGAGCC CATCCCCATC 660
CACTACTGCG CCCCCGCCGG CTTCGCCATC CTGAAGTGCA ACGACAAGAA GTTCAGCGGC 720
AAGGGCAGCT GCAAGAACGT GAGCACCGTG CAGTGCACCC ACGGCATCCG GCCGGTGGTG 780
AGCACCCAGC TCCTGCTGAA CGGCAGCCTG GCCGAGGAGG AGGTGGTGAT CCGCAGCGAG 840
AACTTCACCG ACAACGCCAA GACCATCATC GTGCACCTGA ATGAGAGCGT GCAGATCAAC 900
TGCACGCGTC CCAACTACAA CAAGCGCAAG CGCATCCACA TCGGCCCCGG GCGCGCCTTC 960
TACACCACCA AGAACATCAT CGGCACCATC CGCCAGGCCC ACTGCAACAT CTCTAGAGCC 1020
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ACCATCGTGT TCAACCAGAG CAGCGGCGGC GACCCCGAGA TCGTGATGCA CAGCTTCAAC 1140
TGCGGCGGCG AATTCTTCTA CTGCAACACC AGCCCCCTGT TCAACAGCAC CTGGAACGGC 1200
AACAACACCT GGAACAACAC CACCGGCAGC AACAACAATA TTACCCTCCA GTGCAAGATC 1260
AAGCAGATCA TCAACATGTG GCAGGAGGTG GGCAAGGCCA TGTACGCCCC CCCCATCGAG 1320
GGCCAGATCC GGTGCAGCAG CAACATCACC GGTCTGCTGC TGACCCGCGA CGGCGGCAAG 1380
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ACCAAGGCCA AGCGCCGCGT GGTGCAGCGC GAGAAGCGGG CCGCCATCGG CGCCCTGTTC 1560
CTGGGCTTCC TGGGGGCGGC GGGCAGCACC ATGGGGGCCG CCAGCGTGAC CCTGACCGTG 1620
CAGGCCCGCC TGCTCCTGAG CGGCATCGTG CAGCAGCAGA ACAACCTCCT CCGCGCCATC 1680
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GTGCTGGCCG TGGAGCGCTA CCTGAAGGAC CAGCAGCTCC TGGGCTTCTG GGGCTGCTCC 1800
GGCAAGCTGA TCTGCACCAC CACGGTACCC TGGAACGCCT CCTGGAGCAA CAAGAGCCTG 1860
GACGACATCT GGAACAACAT GACCTGGATG CAGTGGGAGC GCGAGATCGA TAACTACACC 1920
AGCCTGATCT ACAGCCTGCT GGAGAAGAGC CAGACCCAGC AGGAGAAGAA CGAGCAGGAG 1980
CTGCTGGAGC TGGACAAGTG GGCGAGCCTG TGGAACTGGT TCGACATCAC CAACTGGCTG 2040
TGGTACATCA AAATCTTCAT CATGATTGTG GGCGGCCTGG TGGGCCTCCG CATCGTGTTC 2100
GCCGTGCTGA GCATCGTGAA CCGCGTGCGC CAGGGCTACA GCCCCCTGAG CCTCCAGACC 2160

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CGGCCCCCCG TGCCGCGCGG GCCCGACCGC CCCGAGGGCA TCGAGGAGGA GGGCGGCGAG 2220
CGCGACCGCG ACACCAGCGG CAGGCTCGTG CACGGCTTCC TGGCGATCAT CTGGGTCGAC 2280
CTCCGCAGCC TGTTCCTGTT CAGCTACCAC CACCGCGACC TGCTGCTGAT CGCCGCCCGC 2340
ATCGTGGAAC TCCTAGGCCG CCGCGGCTGG GAGGTGCTGA AGTACTGGTG GAACCTCCTC 2400
CAGTATTGGA GCCAGGAGCT GAAGTCCAGC GCCGTGAGCC TGCTGAACGC CACCGCCATC 2460
GCCGTGGCCG AGGGCACCGA CCGCGTGATC GAGGTGCTCC AGAGGGCCGG GAGGGCGATC 2520
CTGCACATCC CCACCCGCAT CCGCCAGGGG CTCGAGAGGG CGCTGCTGTA A          2571
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00325

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N9/00 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 20060 A (THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE; DROPULIC B) 5 June 1997 (1997-06-05) page 58, line 5 - line 24	1-6, 11-22
X	EP 0 759 471 A (HISAMITSU PHARMACEUTICAL CO) 26 February 1997 (1997-02-26) column 5	1-6, 16-22
X	EP 0 612 844 A (ORTHO PHARMA CORP) 31 August 1994 (1994-08-31) column 17 - column 19	1-4, 16-22

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 July 1999	27/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00325

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VANDENDRIESSCHE T ET AL: "Inhibition of clinical human immunodeficiency virus (HIV) type 1 isolates in primary CD4+ T lymphocytes by retroviral vectors expressing anti-HIV genes" JOURNAL OF VIROLOGY, vol. 69, no. 7, July 1995 (1995-07), pages 4045-4052, XP002109322 AMERICAN SOCIETY FOR MICROBIOLOGY US figure 1	1-4, 16-22

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/00325

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 21
is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/GB 99/00325

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9720060 A	05-06-1997	AU 1124997 A	19-06-1997
		CN 1207775 A	10-02-1999
		CZ 9801624 A	16-12-1998
		EP 0871757 A	21-10-1998
		NO 982418 A	27-07-1998
EP 0759471 A	26-02-1997	AU 2419595 A	29-11-1995
		WO 9530755 A	16-11-1995
EP 0612844 A	31-08-1994	AU 5639494 A	01-09-1994
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		NO 940624 A	26-08-1994
		ZA 9401287 A	24-08-1995